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FLUORESCENCE SPECTRA OF BLOWFLY METAXANTHOPSINS

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Abstract—The main visual pigment of blowflies (xanthopsin, Vogt, 1983 *Z. Naturforsch.* **38c**, 329–333) photoconverts into two thermostable metaxanthopsin states M and M′ (e.g. Stavenga *et al.*, 1984 *Photochem. Photobiol.* **40**, 653–659). The fluorescence spectra of the two photoproducts were studied by microspectrofluorometry *in vivo*. The emission spectra of M and M′ are very similar and peak at 660 nm. The excitation spectra of M and M′ have peak wavelengths at $\lambda_{max} \simeq 584$ nm and $\simeq 568$ nm respectively.

INTRODUCTION

The main visual pigment of flies is characterized by a prominent absorption band in the blue-green. This visual pigment type, located in the peripheral visual sense cells R1-6, is converted by light into a thermostable state absorbing in the orange. Formerly, it was assumed that the chromophore of the visual pigment was retinal, hence the fly's visual pigment was called rhodopsin and its photoproduct metarhodopsin (reviews: Hamdorf, 1979; Stavenga and Schwemer, 1984). Recently, however, Vogt (1983) and Vogt and Kirschfeld (1984) established that the chromophore of fly visual pigments is 3-hydroxyretinal. Since this is a xanthophyll derivative, Vogt (1983) proposed to rename the visual pigment xanthopsin, and accordingly the photoproduct metaxanthopsin.

Several studies have focused on the spectral absorption properties of xanthopsin (P) and metaxanthopsin (M) in blowfly, housefly and fruitfly (e.g. Paulsen and Schwemer, 1979; Kirschfeld *et al.*, 1977; Stark and Johnson, 1980). The visual pigment characteristics of these species appear to be virtually indistinguishable.

Since Franceschini (1977, housefly) and Stark *et al.* (1977, fruitfly) performed the first fluorescence observations on fly visual pigments it has become clear that the study of fluorescence is a valuable addition to fly vision research. Whereas the native visual pigment state fluoresces negligibly, the fluorescence of the thermostable photoproduct is substantial. This fact allowed visualization of the distribution of various receptor types in the fly eye (Franceschini *et al.*, 1981a) and, furthermore, yielded the discovery of a new metaxanthopsin-like state, called M′, in the housefly (Franceschini *et al.*, 1981b), having a much greater fluorescence efficiency than M.

A spectral study of M′ has been performed in the housefly by Stavenga *et al.* (1984). Here we report a more extensive study on the fluorescence properties of both M and M′ in the blowfly *Calliphora erythrocephala*, thus extending preliminary studies of Stavenga and Franceschini (1981) and Stavenga (1983).

MATERIALS AND METHODS

Preparation. All experiments were performed on the white-eyed mutant chalky of the blowfly *Calliphora erythrocephala in vivo*. After immobilization with wax, the fly was mounted on the goniometer stage of a microspectrofluorometer.

Apparatus. The microspectro(fluoro)photometer was a Leitz Orthoplan microscope equipped with a NPL 10, 0.20 objective, a Ploemopak 2 fluorescence, incident-light illuminator and a MPV-compact photometer (Hamamatsu R928 photomultiplier) modified to contain an interference wedge (Veril S 200 Schott). In the experiments where the total emission above 665 nm was measured the interference wedge was removed and a Wratten 70 filter (Kodak) was inserted.

Two Xe-lamps, 75 and 450 W, supplied the test and stimulus lights, respectively. Light flux was controlled by neutral density filters (Schott) and shutters (Uniblitz). Monochromatic light was obtained by introducing interference filters (Schott, DAL; halfwidth $\simeq 15$ nm).

Fluorescence was measured from the deep pseudopupil (Franceschini, 1975), i.e. from the superimposed image of the photoreceptors. Thus the fluorescence from about a thousand photoreceptor cells was collected. We note that because the visual pigments of the central photoreceptors R7,8 do not fluoresce in the red (Franceschini, 1977), effects of these minority visual pigments on the excitation spectra may be neglected. The photomultiplier signal was amplified, low-pass filtered (Krohn-Hite 3343) and delivered to a microcomputer (MP200 Data General), which performed data acquisition and processing. All experimental settings in the measurements were under computer control.

Excitation spectra. The excitation spectrum of metaxanthopsin M was determined by measuring the emission above 665 nm induced by a variety of wavelengths. In each case the fly eye was preadapted with blue light to form a maximum amount of metaxanthopsin. Generally, the excitation exposures caused a decreasing metaxanthopsin concentration, and thus a decaying emission signal (Fig. 1, upper traces). The initial value of the experimental curves then was determined by fitting the exponential function $y(t) = a \exp(-t/\tau) + c$. The spectral dependence of the initial value, $y(0) = a + c$, divided by the applied

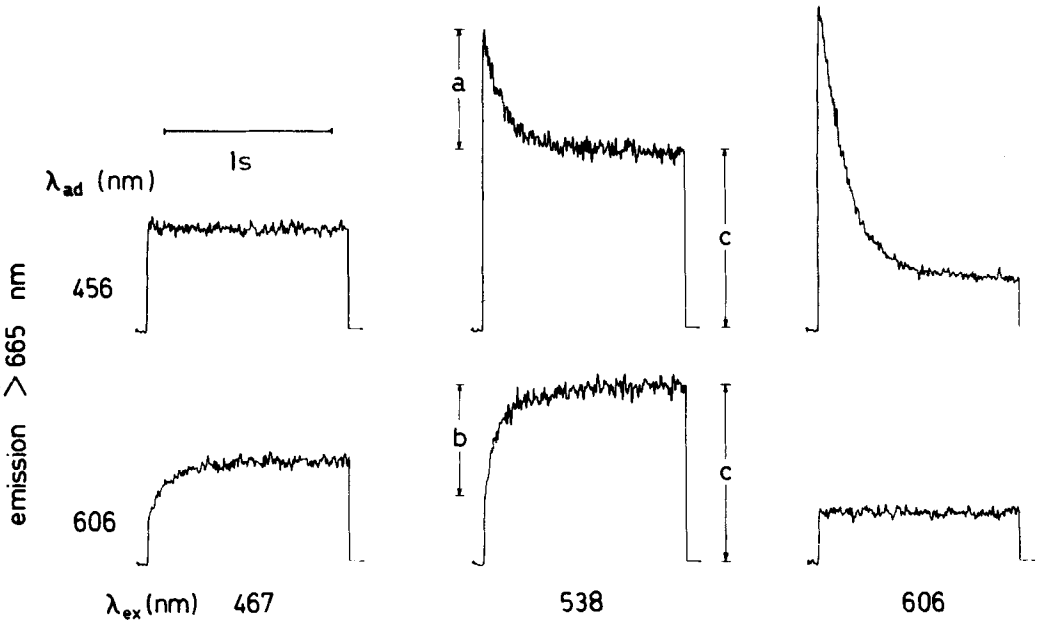

Figure 1. Emission from the deep pseudopupil above 665 nm induced by excitation light of wavelength λ_{ex} = 467 nm (left), 538 nm (middle) and 606 nm (right). Prior to the exposures the eye was adapted to light of wavelength λ_{ad} = 456 nm (upper) and 606 nm (lower traces), respectively. The curves reflect the change in metaxanthopsin content from the photosteady state set by the preadaptation wavelength. (The curves are scaled such that the quantum fluxes at the various excitation wavelengths are identical.) The upper experimental curves are fitted by the exponential function $y(t) = a \exp(-t/\tau) + c$ and the lower curves by $y(t) = -b \exp(-t/\tau) + c$ (see methods).

quantum flux, I, would represent the excitation spectrum if no background fluorescence from non-visual pigments was present. The background contribution could be ruled out by measuring another set of emission curves, starting from a situation with a virtually zero metaxanthopsin content (created by red light preadaptation) (Fig. 1, lower traces). These curves were also fitted with an exponential function $y(t) = -b \exp(-t/\tau) + c$, and thus the background spectrum followed from the initial values ($y(0) = -b + c$), again divided by the quantum flux I. The excitation spectrum (Fig. 2) was then calculated from $(a + b)/I$, and subsequently normalized. A slightly different procedure was followed for determining the excitation spectrum of metaxanthopsin M′ (see RESULTS). The procedures were chosen so that visual pigment bleaching was negligible.

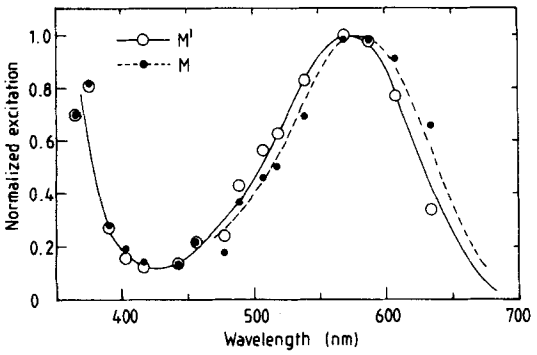

Figure 2. Excitation spectra of metaxanthopsins M (●) and M′ (○). Fluorescence induced by various wavelengths was measured from the deep pseudopupil. Both data sets were fit by an Ebrey–Honig nomogram and normalized at the peak wavelength being 584 nm (M) and 568 nm (M′) respectively.

The quantum flux $I(\lambda)$ delivered to the fly's eye was measured with an EG&G radiometer (type 550-1) or a (EG&G) calibrated HUV 1000B photodiode.

Emission spectra. The emission spectra of M and M′ (Fig. 3) were determined by scanning blue-induced emission above 500 nm from the deep pseudopupil. The spectra were sampled along the wavelength axis at a rate of 4 samples/nm. Background fluorescence, due to non-visual pigments in the fly eye, determined by measuring the fluorescence outside the deep pseudopupil, was subtracted from the deep pseudopupil measurements. The experimentally obtained emission spectra were corrected for the transmission properties of the microscope and the spectral sensitivity of the photomultiplier by dividing these spectra by a spectral scan obtained from a halogen lamp (run at a temperature of 2700 K). The lamp spectrum was calculated from Planck's black-body formula.

RESULTS

The *excitation* spectrum of M was determined by measuring the emission of the blowfly's photoreceptors as a function of the wavelength of the excitation beam (see METHODS). Preadaptation occurred first with 456 nm light. In order to induce a sufficiently large emission signal the excitation light had to be intense. Hence the visual pigment distribution was readily shifted to a new photosteady

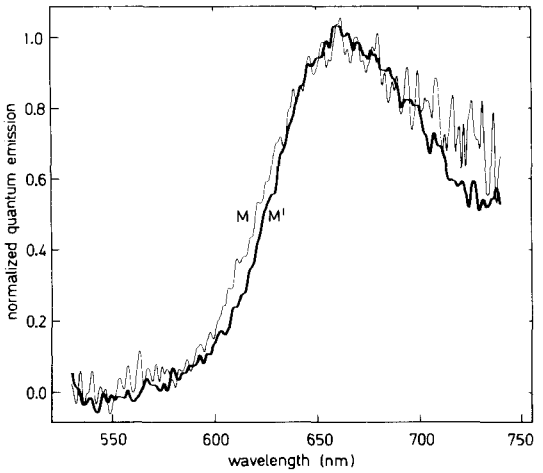

Figure 3. Emission spectra of metaxanthopsins M (thin trace) and M′ (thick trace) induced by blue (467 nm) light. The spectra are both normalized (at ≃ 660 nm).

state determined by the wavelength of excitation (Fig. 1). The initial values of the upper traces originate from the identical (maximal) metaxanthopsin contents, and also include a background fluorescence originating from non-visual pigments in the eye, a dominant part of which are the pigments of the mitochondrial respiratory chain (Stavenga and Tinbergen, 1983; Tinbergen and Stavenga, 1986). The initial values of the lower curves (Fig. 1), obtained after preadaptation with 606 nm light, represent this background fluorescence. After subtracting the background and dividing by the quantum flux (see Methods) the excitation spectrum presented in Fig. 2 (filled circles) resulted. The data were collected from measurements on two flies and each point was measured four times. The datapoints were fit by an Ebrey–Honig nomogram and normalized at the peak wavelength near 584 nm.

The *emission* spectrum of metaxanthopsin (Fig. 3, thin trace) was determined by resolving spectrally the emission from the deep pseudopupil excited by 488 nm light (spectral scan from 500 to 740 nm). The background contribution to this measurement was corrected for as follows. A spectral scan was also made from the fluorescence outside the deep pseudopupil. It appeared that the shapes of the green part ($\lambda<600$ nm) of both spectra (thus within and outside the deep pseudopupil) were spectrally identical. The spectrum obtained from outside the deep pseudopupil was scaled up so that the part in the green equaled that from within the deep pseudopupil. Subsequently the former was subtracted from the latter. The resulting spectrum (Fig. 3) shows that M-emission is maximal at $\lambda \simeq 660$ nm.

Metaxanthopsin (M′)

In housefly visual pigment, high blue intensities populate a state M′ (Franceschini *et al.*, 1981b; Stavenga *et al.*, 1984). The same phenomenon occurs in the blowfly as demonstrated in Fig. 4, which shows the emission above 665 nm induced by alternating blue and red illuminations. In Fig. 4a intense broad-band blue light establishes, within a few ms, a photosteady state with a high metaxanthopsin content. Subsequent red light (Fig. 4b) reconverts the metaxanthopsin back to xanthopsin (compare Fig. 1, top right). Upon prolonged blue irradiation (Fig. 4c) a substantial fluorescence increase is induced, reflecting creation of M′. Clearly this state fluoresces much more strongly than M, as is also seen with red light excitation (Fig. 4d). Furthermore, only prolonged red light produces complete reconversion of M′ (Fig. 4e); the rates of photoconversion of M and M′ back to xanthopsin differ about 3 log units (see also Franceschini *et al.*, 1981b).

Some spectral properties of M′ were reported for the housefly by Stavenga *et al.* (1984). Here we present data for the blowfly. The *excitation* spectrum shown in Fig. 2 (open circles) was determined as follows. M′ was created by exposing the eye for 3 min to broad-band blue light (390–490 nm). Subsequently, the emission above 665 nm induced by various excitation wavelengths was measured. The fluorescence background at each excitation wavelength then was subtracted, using the background determined in the measurements of the excitation spectrum of M. For the derived excitation spectrum measurements from two flies were used and each datapoint was measured four times in total. Again the data were fit by an Ebrey–Honig nomogram and subsequently normalized at the peak wavelength. This peak is located at $\lambda \simeq 568$ nm. The normalization factor was 0.2 times the factor we used for normalizing the excitation spectrum of M, so the fluorescence yield of M′ is five times as high as that of M. The *emission* spectrum of M′ was determined by, again, subtracting the background fluorescence measured from outside the deep pseudopupil. The resulting spectrum, peaking at 660 nm, is shown in Fig. 3 (solid trace).

DISCUSSION

The fluorescence spectra derived for the blowfly metaxanthopsins M and M′ were determined *in vivo* and were only corrected for background fluorescence. The visual pigment is contained within rhabdomeres, long cylindrical structures acting as optical waveguides, and therefore spectral measurements will be affected by the optical inhomogeneities of the fly's eye. The effects on the positions of the excitation and emission maxima will only be slight, however. We estimate that the spectral shapes are not substantially broadened. Of course, measurements of pure spectra require a different approach, namely extraction of the visual pigment which procedure, however, then may change the pigment properties (see Stavenga and Schwemer, 1984).

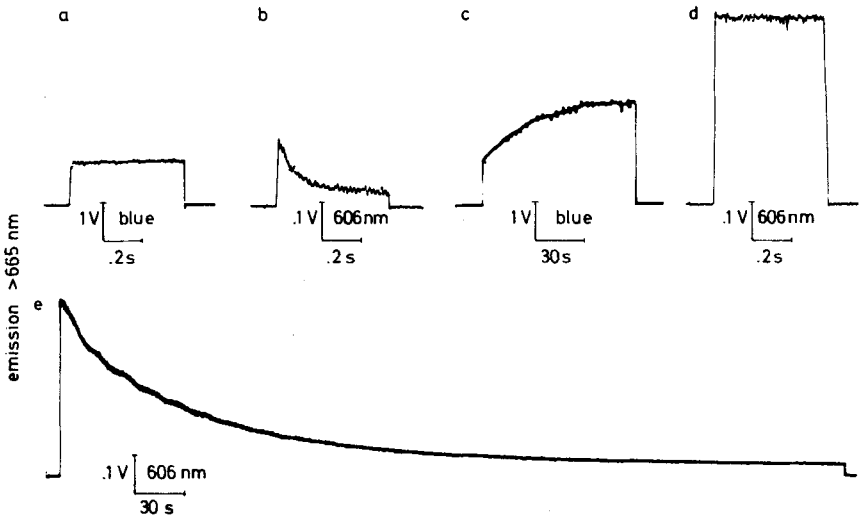

Figure 4. Demonstration of the conversion of visual pigment into the metaxanthopsin M′ state. The fly's eye (*Calliphora chalky*) was preadapted with 606 nm light. A short bright broad-band blue (390–490 nm) flash (a) establishes a high metaxanthopsin content, as shown by the subsequent 606 nm flash (b). However, a long (1.5 min) intense blue exposure (c) creates a high concentration of M′, as is shown by the subsequent 606 nm flash (d), which induces no transient, but only a high plateau. Only prolonged red light (e) can reconvert M′ back into the native xanthopsin state.

From Fig 2 it can be appreciated that the excitation spectra of M and M′ are significantly different and have peak wavelengths of 584 nm and 568 nm respectively. The fluorescence yield of M′ is five times larger than that of M.

The emission spectra of both M and M′ virtually coincide and peak at 660 nm. The blowfly spectra closely correspond to the emission spectra of housefly M′ (Stavenga *et al.*, 1984) and fruitfly M′ (Miller *et al.*, 1984).

Interestingly, the emission spectrum of crayfish metarhodopsin peaks at a very similar wavelength (660–670 nm) whereas its excitation spectrum peaks at 525 nm (Cronin and Goldsmith, 1981), rather different from the blowfly values, 584 and 568 nm respectively. Also, Cronin and Goldsmith (1981) found that changing the pH from 7.5 to 1.9 only slightly (10–20 nm) shifted the emission spectrum of crayfish metarhodopsin to shorter wavelengths, whereas the excitation (and absorption) spectrum was shifted much more strongly: a peak shift from 525 to 470 nm. Hence, the position of the emission spectra seems to be rather independent of the absorption spectra.

Visual pigments when excited from the native state, i.e. with the chromophore in the 11-*cis* configuration, exhibit only a minor fluorescence. Doukas *et al.* (1984) report for both bovine and squid rhodopsin a small fluorescence efficiency: $\phi_f = 1.2\times10^{-5}$. The quantum yield for crayfish metarhodopsin is two orders of magnitude higher: $\phi_f = 1.6\times10^{-3}$ (Cronin and Goldsmith, 1981). Presumably the meta-states of blowfly visual pigment have a similar high yield; at least the native state xanthopsin also fluoresces negligibly compared to metaxanthopsin (e.g. Stavenga and Tinbergen, 1983; see also Govindjee and Ebrey, 1986, and Cronin, 1985). The relatively high fluorescence yield of M′ has proven to be of great value for identification of fly photoreceptors *in situ* (Franceschini *et al.*, 1981a, b).

We may take for granted that the chromophore of M has an all-*trans* configuration, as is common for visual pigment meta-states. However, we hypothesize that the chromophore of M′ is 13-*cis*. This conjecture is suggested by the finding of Ohtsu and Kito (1985) that a 13-*cis*-photoproduct is produced from octopus rhodopsin by irradiation with violet light. In fly M′ is also created by short wavelength light only. We note that bacteriorhodopsin has two distinctly fluorescing states with all-*trans* and 13-*cis* chromophores.

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